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DOI:10.1128/AEM.02121-10

Bacterial endosymbiont localization in *Hyalesthes obsoletus*, the insect vector of Bois Noir in *Vitis vinifera*

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One emerging disease of grapevine in Europe is Bois Noir (BN), a phytoplasmosis caused by '*Candidatus* Phytoplasma solani' and spread in vineyards by the planthopper *Hyalesthes obsoletus* (Hemiptera: Cixiidae). Here we present the first full characterization of the bacterial community of this important disease vector collected from BN-contaminated areas in Piedmont, Italy. Length heterogeneity PCR and denaturing gradient gel electrophoresis analysis targeting the 16S rRNA gene revealed the presence of a number of bacteria stably associated with the insect vector. In particular, symbiotic bacteria detected by PCR with high infection rates in adult individuals, fell within the '*Candidatus* Sulcia muelleri' cluster in the *Bacteroidetes* and in the '*Candidatus* Purcelliella pentastirinorum' group in the *Gammaproteobacteria*, both previously identified in different leafhoppers and planthoppers. A high infection rate (81%) was shown also for another symbiont belonging to the *Betaproteobacteria*, designed as HO1-V symbiont. Because of the low 16S rRNA gene identity (80%) with the closest relative, an uncharacterized symbiont of the tick *Haemaphysalis longicornis*, we propose the new name '*Candidatus* Vidania fulgoroideae'. Other bacterial endosymbionts identified in *H. obsoletus* were related to the intracellular bacteria *Wolbachia pipientis*, *Rickettsia* sp., and '*Candidatus* Cardinium hertigii'. Fluorescent *in situ* hybridization coupled with confocal laser scanning microscopy and transmission electron microscopy showed that these bacteria are localized in the gut, testicles and oocytes. As *Sulcia* is usually reported in association with other symbiotic bacteria, we propose that in *H. obsoletus* it may occur a bipartite or even tripartite relationship between *Sulcia* and *Purcelliella* or *Vidania* or both.

INTRODUCTION

Grapevine yellows are severe insect-borne diseases affecting grape in many wine-producing countries. They are caused by phytoplasmas, cell wall-less bacteria belonging to the class *Mollicutes*, that can multiply in the body of the insect vector and in phloem cells of the host plant (18, 32). An emerging grape yellows is “Bois Noir” (BN), caused by a phytoplasma of the Stolbur group (16Sr-XII), recently proposed as ‘*Candidatus* Phytoplasma solani’ (29). The insect vector of BN is *Hyalesthes obsoletus*, a polyphagous planthopper (Hemiptera, Cixiidae) that can occasionally feed on grapevine despite it is usually found on dicotyledonous weeds (1, 2). A direct approach for controlling BN is not available, but measures for limiting the spread of the disease are based on controlling the insect vector with insecticides and the management of weeds in the vineyard.

The use of biocontrol agents is of increasing interest in pest management (6, 51, 7, 47). One emerging strategy is the “symbiotic control” which implies the exploitation of microorganisms associated to the insect vector to provide anti-disease strategies, such as the reduction of the vector competence (6) or the manipulation of undesirable host traits (47).

For developing a symbiotic control, the identification of dominant symbionts of the insect vector is necessary. In the case of *H. obsoletus*, despite the increasing relevance of BN in European vineyards, only few works have been published describing the microbiota of this insect vector: a preliminary characterization indicating the association with symbionts related to *Wolbachia* and *Bacteroidetes* (23), and a symbiont screening on different planthoppers showing the affiliation of the bacteriome-restricted ‘*Candidatus* Sulcia muelleri’ and ‘*Candidatus* Purcelliella pentastirinorum’ (12). However, the study did not provide details on the localization of symbionts in the insect body.

The present study examined, by means of molecular ecology techniques, the symbiont diversity residing in the body of *H. obsoletus*. We also provide information on the tissue localization of several endosymbionts. The study indicates that several symbionts cohabit the male and female gonads, suggesting that complex interactions between different vertically-transmitted endosymbionts occur in the same insect host (20, 25).

MATERIALS AND METHODS

Insect material and DNA extraction. *H. obsoletus* individuals were collected in 2005-2007 from wastelands close to vineyards affected by BN in Piedmont, North Italy. Ninety-nine individuals of *H. obsoletus* were killed with ethyl acetate and preserved frozen at –20°C or in ethanol until molecular analysis. Twenty *H. obsoletus* adult specimens were dissected to isolate salivary glands, gut, fat bodies, and ovaries. Total DNA of whole insects and dissected organs was extracted according to a method previously described by Doyle and Doyle (19).

Molecular techniques for characterizing the microflora of *H. obsoletus*. Two different molecular methods were used to study the bacterial community associated with *H. obsoletus*. A Length Heterogeneity PCR (LH-PCR) (53, 48) was carried out to screen the diversity of the microbial population associated with *H. obsoletus*. The DNA extracted from insects was subjected to PCR amplification using the eubacterial universal primers 27F and 338R (48); the primer 27F was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) on the 5' end. PCR conditions and sample preparation were performed as previously described (35). LH-PCR fragments were loaded on an ABI Prism 310 capillary electrophoresis system (Applied Biosystems) and run in denaturing conditions using POP-4 running polymer. The LH-PCR data were analyzed with the Genescan 3.1.2 software (Applied Biosystems).

For DGGE analysis (Denaturing Gradient Gel Electrophoresis), the bacterial 16S rRNA genes were amplified by using the forward primer GC357f, containing a 40-bp GC clamp, and the reverse primer 907r, as previously described (50, 35). Polyacrylamide gels (7% of a 37:1 acrylamide-bisacrylamide mixture in 1× TAE buffer) with a gradient of 40-60% or 20-30% denaturant were used; 100% denaturant corresponds to 7M urea and 40% formamide (45).

Sequencing of DGGE bands. Selected DGGE bands were excised from the DNA eluted and used as template in PCR reamplification reactions with the primers 357F (without GC clamp) and 907R, performed as previously described (35). The obtained PCR products were purified and sequenced (Primm, Milan, Italy), resulting sequences were compared with the sequence database at the National Center for Biotechnology Information (NCBI) using BLAST (http://www.ncbi.nlm.nih.gov/blast) (3).

Based on the sequences of DGGE bands corresponding to '*Ca.* Phytoplasma solani', *Wolbachia*, *Cardinium*, '*Ca.* Sulcia muelleri', '*Ca.* Purcelliella pentastiridorum', and HO1-V symbiont, additional sequences of the 16S rRNA gene of these microbes outside the 5'- and 3'- ends of the DGGE fragments were obtained by performing specific PCR reactions with primer pairs previously reported or designed for this work, as shown in Table 1. The six forward primers were used in combination with the eubacterial reverse primer 1495R, while the six reverse primers were coupled with the forward universal primer 27F (35). Therefore, the flanking regions at the 5'- and 3'- end of the DGGE fragments of these bacteria were obtained.

After amplification and sequencing, all of the obtained 16S rRNA sequences were subjected to BLAST analysis and aligned with the corresponding 16S rRNAs of close relatives and with other unrelated eubacterial sequences. Alignments were performed by using the software available in the Ribosomal Database Project website (14). Phylogenetic analyses were performed by using Jukes and Cantor distance estimation with the TREECON 1.3b package (56). A 50% majority rule bootstrap consensus tree (1000 replicates) was generated. Gaps were treated as a fifth base.

Detection of the prevalence of *H. obsoletus* – associated microorganism populations by means of PCR. We examined by means of specific PCR screenings the abundance of six bacteria present in *H. obsoletus* ('*Ca.* Phytoplasma solani', *Wolbachia*, *Cardinium*, '*Ca.* Sulcia muelleri', '*Ca.* Purcelliella pentastiridorum', and HO1-V symbiont). Such microorganisms were considered of particular interest because either they were well-known for their functions in other insect models, or they appeared extremely abundant the in the diversity screenings. The analyses were performed on 80 insect specimens, including those examined by DGGE and LH-PCR. Seventy individuals (28 females and 32 females) were used for whole-insect DNA extraction. Ten individuals (females and males, 5 each) were dissected and DNA was extracted from the organs (fat bodies, gut, ovaries, testes, and salivary glands). To evaluate the prevalence of '*Ca.* Phytoplasma solani', specific PCR reactions were performed using the primer pair M1/P8 (34) or the BN F/R primer pair (5). The *wsp* gene of *Wolbachia* was amplified using the *wsp*81F and *wsp*691R primer pair as previously described (10).

The alignments of the *Sulcia*, *Purcelliella*, and HO1-V symbiont 16S rRNA sequence with related bacterial sequences were used to design primer pairs specifically targeting the symbionts (Table 1). Selected primers for *Sulcia* were SF1 (positions 656-677 *E. coli* strain K-12) and SR (positions 839-862 *E. coli* strain K-12), and amplified a 185 bp fragment. They did not match with any bacterial or invertebrate sequences in GenBank at the time of checking; moreover they matched with the cixiid- associated *Sulcia* sequences (FN428791 and FN428795). Selected primers for *Purcelliella* were PF (positions 472-496 *E. coli* strain K-12) and PR1 (positions 855-876 *E. coli* strain K-12) and amplified a 404 bp fragment. They matched with the described *H. obsoletus*- associated '*Ca.* Purcelliella pentastiridorum' sequence (FN428799), but not with other cixiid-associated *Purcelliella* sequences (FN428803); furthermore they did not correspond to any bacterial or invertebrate sequences in GenBank at the time of checking. Selected primers for HO1-V symbiont were VF1 (positions 161-182 *E. coli* strain K-12) and VR (positions 427-446 *E. coli* strain K-12) and amplified a 285 bp fragment. They did not coincide with any bacterial or invertebrate sequences in GenBank at the time of checking. Each PCR assay included a cloned amplicon sample specific for each microorganism as positive control and a water sample as negative control. Amplifications were performed with the following conditions: an initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C (when using SF1-SR primer pair) or 55°C (when using PF-PR1 or VF1-VR primer pairs), 1 min at 72°C and a final extension step of 7 min at 72°C. As control a sample of the PCR products obtained from each specific PCR reaction was sequenced.

A PCR screening with the VF/R primer pair was also carried out on DNA samples of whole-body insects of the species *Hyalesthes luteipes*, *Reptalus cuspidatus* and *Reptalus melanochetus*, in order to assess the distribution of these symbiont among other cixiids.

Localization of symbionts in *H. obsoletus* by means of TEM and FISH. Twenty-three individuals (5 females, 5 males and 13 nymphs) were dissected and prepared to be studied with transmission electron microscopy (TEM), as previously reported(8). Thin sections (80 nm) were examined under a Zeiss EM900 transmission electron microscope. Fluorescent *in situ* hybridization (FISH) was performed on 25 *H. obsoletus* individuals (10 females, 10 males and 5 nymphs) to observe the distribution of the phytoplasma, *Cardinium*, *Wolbachia, Sulcia*, *Purcelliella* and the HO1-V symbiont within the insect body. Specific fluorescent probes targeting the 16S rRNA gene were used (Table 1). Hybridization of *Wolbachia* was performed by using the probes W1 and W2 (27), while for the specific hybridization of the other bacteria we designed the following probes: ph1107 for the phytoplasma; card172 and card1069 for *Cardinium*; S1150 for *Sulcia*; P820 for *Purcelliella*, V370 for the HO1-V symbiont. We also used the probes MCP52 (55) , matching with portions of 16S rRNA of different *Mollicutes*, CFB319 (42), targeting the 16S rRNA gene of *Bacteroidetes*, and EUB338 (4), matching with 16S rRNA of all Eubacteria. Probes card172 and card1069, S1150, and W1 were labeled at the 5' end with the fluorochromes Cy3 (indocarbocyanine, absorption/emission at 550/570 nm) or Cy5 (indodicarbocyanine, absorption/emission at 650/670nm); probe ph1107 was labeled with Texas Red (TR, absorption/emission at 595/620 nm); probes P820 and V370 were labeled with HEX (4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein, absorption/emission at 535/556 nm), 6-JOE (6-carboxy-4',5'-dichloro-2', 7'-dimethoxy fluorescein, absorption/emission at 520/548 nm) or ROX (Carboxy-X-rhodamine, absorption/emission at 580/600 nm), and probes W2, MCP52, CFB319, and EUB338 were labeled with fluorescein isothiocyanate (FITC, absorption/emission at 494/520 nm). Insects were dissected to collect salivary glands, guts and gonads. Paraformaldehyde-fixed insect dissections were hybridized according with the method described by Crotti et al. (15).

Nucleotide sequence accession number. The nucleotide sequences of '*Ca.* Sulcia muelleri', '*Ca.* Purcelliella pentastiridorum' and the HO1-V symbiont's 16S rRNA genes were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the following accession numbers: FM992371 (*Sulcia*), FR686933 (*Purcelliella*), FR686932 (HO1-V symbiont of *H. obsoletus*), and FR733652 (Betaproteobacterial symbiont of *R. melanochetus*).

RESULTS AND DISCUSSION

Characterization of the bacterial community associated with *H. obsoletus*. The bacterial community associated to *H. obsoletus* from BN-contaminated areas was studied by means of LH-PCR. The screened insects showed some dominant peaks (e.g. peaks at 338, 343, 361 bp) that were conserved in almost all the tested individuals, suggesting that certain bacterial species have a stable association with *H. obsoletus* (Fig. 1A). Other peaks (e.g. peaks at 333, 342 and 349 bp) were found only in few insects indicating an occasional association. To identify the taxonomic affiliation, we amplified a portion of about 600 bp of the 16S rRNA gene from the total DNA of the insects and separated the amplified fragments by means of DGGE (Fig. 1B). Although the community profiles of different individuals showed some variability, certain bands were rather conserved in the individuals. DGGE experiments, performed with different denaturing gradient conditions, permitted to recover some other bands associated with few insects (Fig. 1C).

The sequences obtained from the bands isolated from DGGE gels are presented in Table 2 along with the closest relatives found in the RDP database. Band A1 was found in most of the tested individuals (83%), and showed a 99% sequence identity with the *Bacteroidetes* '*Ca.* Sulcia muelleri'. This bacterium was firstly reported as the 'a-symbiont' of Auchenorryncha by Müller (44), and then it was described by Moran *et al.* (42) as a novel clade of strap-shaped *Bacteroidetes* that harbor a small genome and are associated with both Cicadomorpha and Fulgoromorpha. '*Ca.* Sulcia muelleri' has been recently reported in association with some cixiids (12). The almost entire sequence of the 16S rRNA gene of this bacterium grouped in a branch of the neighbor-joining phylogenetic tree including *Sulcia* symbionts of Fulgoromorpha insect hosts belonging different families (Fig. 2A). The phylogenetic analysis confirmed the strong congruency between the phylogeny of the symbiont and that of its host reported for all the '*Ca.* Sulcia muelleri' previously described (41, 54).

Band A2 was found in half of the tested individuals and showed a 100% identity with *Wolbachia pipientis*, an intracellular reproductive manipulator previously described in different insect models including leafhoppers (59, 52, 20, 16). The almost entire 16S rRNA gene of this symbiont was obtained by combining in PCR experiments the newly-designed primers WF and WR specific for *Wolbachia* and bacterial universal primers (data not shown). The sequence was phylogenetically affiliated within the *Wolbachia* supergroup B.

Band A3 and B1 were, respectively, 99 and 88% similar to the 16S rRNA gene of an endosymbiont of the mite *Oppiella nova*, affiliated to the genus '*Ca.* Cardinium' within the *Bacteroidetes*. *Cardinium* includes endosymbionts infecting numerous arthropods and able to induce multiple reproductive effects in their hosts (63, 64 58, 59). The bands of *Cardinium* were detected in 50% (12 of 24) of the individuals examined by means of DGGE. *Cardinium* was detected in all the individuals that showed the presence of *Wolbachia*. To acquire the almost complete 16S rRNA gene sequence of this endosymbiont, *Cardinium*-specific primers (35) were combined with universal primers. The obtained sequence was affiliated with *Cardinium* endosymbionts of several mite and insect species (data not shown).

Band C1, observed in 75% of tested individuals, showed a 100% sequence similarity with '*Ca*. Purcelliella pentastirinorum', a *Gamma-3proteobacterium* recently described as one of the bacteriome- associated symbionts of several cixiid species (12). Evolutionary studies on this bacterium showed it is restricted to the tribe Pentastirini, and it contributed to the diversification of this tribe within the Fulgoromorpha (12). The almost entire 16S rRNA sequence of this bacterium, obtained by combining specific and universal primers, was incorporated in the branch of the *Gammaproteobacteria* phylogenetic tree that includes *Purcelliella* symbionts of the genus *Hyalesthes* and other cixiids (Fig. 2B), confirming the high congruency between symbiont and host.

Furhermore, band C2, which was repeatedly found in the specimen tested by means of DGGE, did not show any significant affiliations based on sequence similarity, and had an uncultured *Betaproteobacterium* associated to the bush tick *Haemaphysalis longicornis* (46) as the closest relative with a 79% sequence similarity, while the nearest determined organism was the Neisseriales *Kingella kingae* (GenBank AY551998). The genus *Kingella* includes human pathogens responsible for several paediatric infective diseases (62). The almost entire sequence of the 16S rRNA gene, named HO1-V, grouped in a separated branch, together with its tick-associated closest relative, within the phylogenetic tree that includes different orders of symbiotic and free-living *Betaproteobacteria*, neighboring to Neisseriales and Burkholderiales (Fig. 3). The extremely low level of sequence identity even with the closest relatives suggests the HO1-V symbiont is quite different from the nearest previously reported organisms. It is interesting to point out that this bacterium is one of the few examples of major symbionts belonging to the beta subclass in the *Proteobacteria*, while primary symbionts fit more commonly in the *Gammaproteobacteria* or the *Bacteroidetes* groups (43).

Other bands were found only in certain individuals with a low prevalence. For instance, band A4 showed a 99% sequence identity with '*Ca.* Phytoplasma solani'. By combining primers specifically designed for sequence A4 with bacterial universal primers, the almost full 16S rRNA gene sequence of the pathogen was obtained and phylogenetically affiliated to that of the Bois Noir Phytoplasma (data not shown). The detection of the Bois Noir phytoplasma by DGGE suggests that the titer of this pathogen is much higher than that of other phytoplasmas such as the Flavescence dorée phytoplasma, which has never been detected by means of PCR-DGGE in the total DNA extracted from insect (35).

In few individuals, a sequence with a 99% identity with *Rickettsia limoniae*, corresponding to bands A5 and B2 in the PCR DGGE gels of Fig. 1, has been detected. Bacteria of the genus *Rickettsia* are human and animal pathogens vectored by blood-feeding insects. Nevertheless members of this genus were found in association with the pea aphid *Acyrthosiphon pisum* (13) and with the whitefly *Bemisia tabaci* (25) and bacteria of the species *R. limoniae* have been identified in the cranefly *Limonia chorea* (GenBank AF322443).

Band B3 in Fig. 1C, corresponding to a sequence strictly related to the *Bacteroidetes* *Chryseobacterium joostei*, has been detected only in one individual.

All the other bands detected by DGGE (Fig. 1B) did not give readable sequences.

Prevalence and localization of the main symbionts of *H. obsoletus*. The prevalence of '*Ca.* Phytoplasma solani', *Wolbachia*, *Cardinium*, '*Ca.* Sulcia muelleri', '*Ca.* Purcelliella pentastiridorum', and HO1-V symbiont in *H. obsoletus* was studied by PCR assays targeting the 16S rRNA gene with symbiont-specific primers (Table 3).

In whole insects, '*Ca.* Phytoplasma solani' showed an average infection rate of 17.5%, with 22.6% females and 12.8% males that were found to be positive. These values are in agreement with previous reports (33, 2, 11).

While the *Cardinium* symbiont was found in 38.8% of the checked insect population, with a slightly lower incidence in the whole-body females (35.5%) than in males (43.6), the minimal infection rate of *Wolbachia* was in average 60%. The symbiont was found more frequently in females (74.2% positive insects) than in males (51.3% positive individuals). This *Alphaproteobacterium* has been reported in all the major orders of insects, with a variable infection rate at a specific level, from about 20% to more than 50% (60, 30, 59).

The minimal infection rate of *Sulcia* and of the HO1-V symbiont were similar, 85% and 83.8% of the samples respectively. Also the distributions of the two symbionts in males and females were comparable, with 90.3% *Sulcia*-infected and HO1-V-infected females, and 82% *Sulcia*-infected and 84.6% HO1-V-infected males. Moreover, *Purcelliella* was present in 67.5% of tested specimens, with a minimal infection rate of. 74.4% in females and of 66.7% in males.

The presence of the HO1-V symbiont was detected also in the cixiid species *H. luteipes*, *R. cuspidatus* and *R. melanochetus*, with infection percentages of 70% (7/10), 30% (3/10) and 40% (4/10), respectively.

A first insight in the localization of the symbionts was provided by specific PCR screenings on dissected body parts, as summarized in Table 3. All of the bacteria were found in the intestines and (with lower infection rates) in salivary glands; in the gonads we detected almost all of the microbes with a few exceptions: we were not able to find the phytoplasma in both male and female gonads, and *Wolbachia* was observed only in the ovaries and not in testes.

A more detailed localization of the bacteria associated to *H. obsoletus* was provided by FISH experiments. The localization of '*Ca.* Phytoplasma solani' was firstly explored using a *Mollicutes*-specific probe. By dissecting salivary glands, it was possible to identify the different lobes and visualize the gland ducts that release saliva during feeding (Fig. 4A, B). Positive hybridization signals were observed in one of five individuals tested (Fig. 4B). Signals were particularly concentrated in the duct of the salivary gland suggesting that the phytoplasmas actively multiply in the salivary duct before the injection in the plant (Fig. 4B, C). To confirm such results, a Stolbur-specific probe was designed and used on dissected salivary glands, where a neat amplification signal was observed in the whole gland lobe (Fig. 4D, E).

Despite in different leafhoppers and planthoppers '*Ca.* Sulcia muelleri' has been observed in the typical position in the bacteriome, in none of the dissected specimen we were able to observe and isolate the specific organ. On the other hand, the localization of *Sulcia* in the body of *H. obsoletus* was studied in the gut, the salivary glands, and the female and male gonads. All of these organs except salivary glands showed a massive presence of the symbiont. By using a specific probe, *Sulcia* appeared associated to the entire gut (Fig. 5A-D), with a denser cell concentration in certain portions of the interior of the gut (Fig. 5A). When observed at higher magnifications (Fig. 5D) the symbiont appeared in clusters of strap-shaped cells previously described as typical of '*Ca.* Sulcia muelleri' (42). In addition, close to the intestinal wall, cells of Bacteroidetes other than *Sulcia* were found. It can be presumed that these bacteria are referable to *Cardinium*, the only other Bacteroidetes massively represented in *H. obsoletus.*

Examination of the gonads of *H. obsoletus* by means of FISH with the probe specific for *Sulcia* showed the bacterium associated both to the ovary (Fig. 6 A-F, L) and the testicles (Fig. 6N-R). By comparing FISH with the universal probe for bacteria and a specific probe for *Sulcia* in an entire ovary it was possible to find signals for the symbiont in all the ovarioles (Fig. 6D) but not in the ovary duct where other bacteria were resident (Fig. 6C). *Sulcia* appeared associated to the oocytes and the nurse cells (Fig. 6F), but not to the follicular cells of the ovariole, where other bacteria were detected by using a universal probe for bacteria (Fig. 6E). A more accurate analysis of the ovary with a TEM showed at least three different cell morphologies associated to the oocyte and the follicular cells (Fig. 6I-J). While bacterial cells in the oocyte (Fig. 6I) showed the distinctive strap shape of '*Ca.* Sulcia muelleri' (42), some bacteria in the cytoplasm of the follicular cells are probably *Wolbachia,* confirming the hybridization signal observed in the follicles when using the *Wolbachia*-specific probe (Fig. 6G-H). In addition, other bacteria were also observed in the follicle cell cytoplasm with the brush-like structure of '*Ca.* Cardinium hertigii' (Fig. 6I-J) typical of these maternally-transmitted endosymbionts (35, 64, 65). Within the male gonads, *Sulcia* was specifically associated with testicles but not with other organs (Fig. 6N-R). FISH with the universal probe Eub338 showed that bacteria other than *Sulcia* specifically colonize the accessory glands of the male gonads (Fig. 6N). These bacteria could be the HO1-V symbiont, as hybridization of *H. obsoletus*'s male gonads with the specific probe for this microorganism gave a strongly positive result both in testicles and in accessory glands, while only a weak signal was obtained by FISH on organs other than the testicles with the *Sulcia*-specific and *Purcelliella*-specific probes (Fig. 6O-R).

'*Ca.* Sulcia muelleri' is typically associated with another bacterial symbiont that varies among insect groups: in sharpshooters it is coresident with the *Gammaproteobacterium* '*Ca.* Baumannia cicadellinicola' (41); in cicadas it is associated with the *Alphaproteobacterium* '*Ca*. Hodkinia cicadicola' (38); in spittlebugs its cosymbiont is the *Betaproteobacterium* '*Ca*. Zinderia insetticola' (37). In all of these systems, both the symbionts provide essential nutrients to the host, and are nutritionally interdependent with each other (39, 36, 37). We cannot exclude that the HO1-V symbiont is the complementary symbiont of *Sulcia*. Indeed, although we do not have knowledge on the possible co-localization within the same bacteriome of *Sulcia* and the HO1-V symbiont, we observed both the *Bacteriodetes* and the *Betaproteobacterium* within the ovaries or eggs, implying they are maternally transmitted together. This suggests the two symbionts could have undergone a co-evolution, with the possible development of complementarity.

The distribution in *H. obsoletus* of *Purceliella*, previously known to be in the bacteriome as well as *Sulcia*, was studied in salivary glands, guts, and male and female reproductive systems. A positive hybridization signal was present in the salivary glands (data not shown) and in the gut (Fig. 5G, H). Nevertheless, we were not able to observe any detectable fluorescence neither in ovaries nor in ovaric eggs, while a weak hybridization signal was present in the male gonads (Fig. 6Q).

Hybridization with the probe specific for the HO1-V symbiont was firstly performed on the insect gut, where a heavy signal was detected, indicating considerable amount of bacterial cells residing in this organ (Fig. 5F). Also ovaric tissues and oocytes (Fig. 6K, L), together with male gonads (Fig. 6R), were observed to host the HO1-V symbiont. On the contrary, no hybridization signal was visible in *H. obsoletus* salivary glands.

Cardinium is known to be associated with several reproductive disorders, including parthenogenesis in parasitoid wasps of the genus *Encarsia* (64), feminization in the mite *Brevipalpus phoenicis* (57) and cytoplasmic incompatibility in *Encarsia pergandiella* (28). It localizes in different organs and tissues of insect hosts (65, 31, 49), including follicle cells of ovaries, as well as oocytes and nurse cells (65) as observed in *Encarsia* spp. To evaluate the localization of this *Bacteroidetes* in the body of *H. obsoletus*, hybridization with the specific probes was firstly carried out on salivary glands and gut. No successful hybridization was obtained in the first, while in the digestive tube a massive signal was detected (Fig. 5E). This symbiont was detected also in the ovaries, with a specific localization in the follicle area, confirming what observed by means of TEM, and in the male gonads (data not shown). These data would suggest a peculiar localization pattern for *Cardinium*-*Sulcia* in *H. obsoletus*, with *Sulcia* in the oocytes and *Cardinium* in the follicle cells; nevertheless we were not able to define a precise localization within the gonad tissues of the HO1-V symbiont.

FISH using a *Wolbachia*-specific probe showed the bacterium associated to the female oocytes and in the mature eggs (Fig. 6G-H, Fig. 7A-C). Hybridization signals were found also in the gut of nymphs (Fig. 7D, E). The localization of *Wolbachia* in different tissues of female gonads of *H. obsoletus* suggests also for the planthopper the vertical transmission pattern reported for insect hosts of this bacterium.

As reported for several mite and hymenopteran species we observed in *H. obsoletus* a double infection of both the sexual manipulators *Cardinium* and *Wolbachia*. (58, 64, 21, 24). The presence in gonads of both these potential sexual manipulators opens new perspectives for the investigation of possible reproductive abnormalities such as sex-ratio alterations and way of action and interference between sexual symbionts.

Overall considerations. Our investigations on the bacterial diversity associated with *H. obsoletus* indicated that several bacterial species inhabit the insect body, revealing a complex symbiotic organization. Some of the bacterial symbionts were related to bacteria previously described as reproductive manipulators, such as *Wolbachia* and *Cardinium*; others, like *Sulcia* and *Purcelliella*, were proved to be primary symbionts of different Auchenorrhyncha, often involved in the host's nutrients supply (61, 36, 39, 12). Indeed such bacteria were found in almost all the individuals, suggesting they could play important - if not essential - roles for the host. The high infection rate of the HO1-V symbiont suggests that also this bacterium has a strict association with its host. Although we do not have knowledge on the possible role of this microorganism in the insect biology, we can suppose a major function.

All of these bacteria were widely distributed within the insect body, massively colonizing different organs, especially gut and male and female gonads. Interestingly, in the gonads the symbionts were detected in both oocytes and testicles; this may suggest a venereal transmission from male to female, as reported for beneficial symbionts in aphids (40) and for the acetic acid bacterium *Asaia* sp. in *Anopheles stephensi* (22, 17).

Potential interactions between bacteria co-localized in the host tissues, particularly in the gonads, should be deeply investigated in the future. The elucidation of the role of these microorganisms in the host could be useful in a symbiotic approach to control phytoplasmoses either with the expression of antagonistic factors by microorganisms cross-living with the phytoplasma, or by means of reproductive manipulators helping to drive the establishment of antagonistic symbionts or to imbalance natural populations of the planthopper with the final aim of limiting BN diffusion.

The low 16S rRNA gene identity of the HO1-V symbiont with the closest relative (80%), which moreover is an uncultured organism, supports the proposal of a novel clade of symbionts of cixiids. Indeed the HO1-V symbiont is strongly associated to *H. obsoletus*, moreover at least other 3 cixiid species (one in the genus *Hyalesthes* and two of the genus *Reptalus*) were shown to host this bacterium for which we propose the new name '*Candidatus* Vidania fulgoroideae'. The generic name honors Carlo Vidano, an Italian auchenorrhynchologist of the University of Turin who first described and studied the biology of phytoplasma vectors in Italy. The species name refers to the superfamily Fulgoroidea, which includes the family of *H. obsoletus* harboring the symbiont. Distinctive features of '*Ca.* Vidania fulgoroideae' are the following unique 16S rRNA gene sequences (positions according to homologous *E. coli* positions): ACA ATC AAA TAT GCC TTT TGA AAA GGG ATT TTA AAT TCT TTA TAA AGT TAT ATT TAA AAA TAT AAT AAA ATG GAC TTA TTA AAT AAA TTA TGT TTT AA (positions 133 to 231), GAT GAA GGT TGA TAA GAT CGT AAA ACA CTT TTT TTA ATT AAT AAA AAC TTG TAT AAA (positions 427 to 484), AGT TTT TAA CTT ATC ATA AAA GGA CCG CTA AAA ATA TAA AAA (positions 1139 to 1181), and TTT TTA CAG CGA GTA AAT AAG CTG A (positions 1254 to 1279).

ACKNOWLEDGMENTS

We are grateful to the Centro Interdipartimentale Grandi Strumenti of the University of Modena and Reggio Emilia for confocal microscopy analysis. Partial financial contribution comes from the Italian Ministry for Research (MIUR), within the project PRIN 2007 "Caratterizzazione del microbiota associato a *Scaphoideus titanus* e *Hyalesthes obsoletus*, cicaline vettrici di fitoplasmi nella vite ed isolamento e studio della localizzazione di batteri acetici simbionti" and the European Union in the ambit of project BIODESERT (European Community's Seventh Framework Programme CSA-SA REGPOT-2008-2 under grant agreement n^{o} 245746). E.C., C.B. and D.D. benefited of travel grants from Cost Action

FA0701: “Arthropod Symbiosis: From Fundamental Studies to Pest and Disease Management”.

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Table 1. Oligonucleotides adopted in this work to obtain the almost entire 16S rRNA gene sequences of the symbionts, for prevalence screenings and for FISH analyses. The sequences of oligonucleotides designed and published in previous studies are not reported.

Target organism	Primer pair (sequence 5'-3')		Probe (Fluorochrome-sequence 5'-3')
	Forward	Reverse	
'*Ca.* Phytoplasma solani'	PhF (CTAAACAGTTTTCATAGCATCACAA)	PhR (TTGTGATGCTATGAAAACTGTTTAG)	ph1107 (TR-GATGGCAATTAACAACAAGGGT)
Wolbachia	WF (TTAAATATGGGAAGTTTACTTTCTGTATTAC)	WR (GTAATACAGAAAGTAAACTTCCCATATTTAA)	W1 (27) W2 (27)
Cardinium	EndoF1(35)	EndoR3(35)	card172 (Cy3-ATCTTTCTAGCATGCGCTAA) card1069 (Cy3-GCACCTTGTATTCCGTCC)
'*Ca.* Sulcia muelleri'	SF (ATMTAGACAKAAAATATTCAGTG) SF1 (AGATAGGAGTAACTGGAATGT)	SR (CACTGAATATTTTMTGTCTAKAT)	S1150 (Cy3-ACATTCCAGTTACTCCTATCT)
'*Ca.* Purcelliella pentastiridorum'	PF (GTATTTTATTAATAATAAAATATG)	PR (CATATTTTATTATTAATAAAATAC) PR1 (AGAAAACACGGCAAAATCACC)	P820 (HEX / 6-JOE / ROX-AGAAAACACGGCAAAATCACC)
HO1-V symbiont	VF (GATGAAGGTTGATAAGATC) VF1 (TTTTAAATTCTTTATAAAGTT)	VR (GATCTTATCAACCTTCATC)	V370 (HEX / 6-JOE / ROX-GATCTTATCAACCTTCATC)
Mollicutes			MCP52 (55)
Bacteroidetes			CFB319 (42)
Eubacteria	27F (9)	1495R (9)	EUB338 (4)
'*Ca.* Baumannia cicadellinicola'			Pro319 (42)

Table 2. Identification of microorganisms associated to *H. obsoletus* according to DGGE profiles in Fig. 1.

Band ID	Most related species	Accession No.	Identity (nt%)	Putative classification	No. positives[a]
A1	'*Candidatus* Sulcia muelleri'	DQ066627	99% (525/528bp)	*Bacteroidetes,* Flavobacteriales	16/18
A2	*Wolbachia pipientis*	DQ235291	100% (488/488bp)	*Alphaproteobacteria*; Rickettsiales	9/18
A3	Endosymbiont of *Oppiella nova*	AY279414	99% (515/520bp)	*Bacteroidetes*	11/18
A4	'*Candidatus* Phytoplasma solani'	DQ222972	99% (505/506bp)	*Mollicutes*, Achioleplasmatales	3/18
A5	*Rickettsia limoniae*	AF322443	99% (503/508bp)	*Alphaproteobacteria*; Rickettsiales	4/18
B1	Endosymbiont of *Oppiella nova*	AY279414	88% (362/410bp)	*Bacteroidetes*	1/6
B2	*Rickettsia limoniae*	AF322443	99%(494/498bp)	*Alphaproteobacteria*; Rickettsiales	1/6
B3	*Chryseobacterium joostei*	AY466722	100%(529/529bp)	*Bacteroidetes,* Flavobacteriales	1/6
C1	'*Candidatus* Purcelliella pentastirinorum'	FN428799	100%(543/543bp)	*Gamma-3Proteobacteria*	15/18
C2	*Haemaphysalis longicornis*- associated microorganism	AB001520	79%(443/556)	*Beta Proteobacteria*	14/18

[a] Number of individuals positive for the presence of the specific band in DGGE analysis compared to the total number of individuals analyzed

Table 3. Prevalence of symbionts in different organs or tissues of *H. obsoletus* as determined with specific PCR assays. A total of 80 individuals has been used in the assays, including 36 females and 44 males. Seventy individuals were used as whole insects, while 10 were used for dissecting the different organs.

Symbiont	Whole insect[a]	Gut[a]	Ovaries[a]	Testes[a]	Salivary glands[a]
Sulcia	60/70 (F, 28/31; M, 32/39)	8/10 (F, 4/5; M, 3/5)	4/5	2/5	2/10 (F, 1/5; M, 1/5)
Wolbachia	43/70 (F, 23/31; M, 20 /39)	5/10 (F, 5/5; M, 0/5)	3/5	0/5	2/10 (F, 2/5; M, 0/5)
Cardinium	28/70 (F, 11/31; M, 17/39)	3/10 (F, 2/5; M, 1/5)	1/5	2/5	3/10 (F, 2/5; M, 1/5)
Purcelliellia	50/70 (F, 24/31; M, 26/39)	3/10 (F, 2/5; M, 1/5)	3/5	1/5	2/10 (F, 2/5, M, 0/5)
HO1-V	61/70 (F, 28/31; M, 33/39)	4/10 (F, 3/5; M, 1/5)	4/5	1/5	3/10 (F, 1/5; M, 2/5)
Ca. P. solani	12/70 (F, 7/31; M, 5/39)	2/10 (F, 1/10; M, 1/10)	0/5	0/5	1/10 (F, 1/5; M, 0/5)

[a] Number of individuals positive in specific PCR assay over the total number of tested individuals. M, males; F, females.

Fig. 1. Bacterial diversity associated to *H. obsoletus*. A) Example of LH-PCR profiles of whole insects collected in 2005-2007 from uncultivated areas in Piedmont, Italy. Numbers refer to different individuals tested. B) and C) DGGE profiles, in 7% polyacrylamide gels with 20 to 40% (B) and 30 to 50% (C) denaturation gradients, of partial 16S rRNA bacterial genes amplified from DNA extracted from whole insects collected in 2005-2007 from wastelands in Piedmont, Italy. Numbers over the lanes refer to the tested individuals. The identity of sequences of bands marked with arrows is given in Table 1 according to the band ID (A1-A5 and B1-B3).

Fig. 2. Phylogenetic affiliation of the almost entire 16S rRNA gene of bacteria associated to *H. obsoletus*. A) '*Ca.* Sulcia muelleri'. Clades of insect hosts of *Sulcia* symbionts are shown. B) '*Ca.* Purcelliella pentastirinorum'. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. The scale bar represents the sequence divergence.

Fig.3. Phylogenetic position of the nearly full 16SrRNA gene of the HO1-V symbiont. Orders within the *Betaproteobacteria* are indicated. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. The scale bar represents the sequence divergence.

Fig. 4. Localization of phytoplasma cells in the salivary glands of *H. obsoletus*. A) Interferential contrast micrograph showing different lobes of the salivary gland. The salivary ducts, indicated by arrows, are visible in certain lobes. B) CLSM image of FISH of the salivary gland lobe identified in A) with an asterisk, hybridized with the *Mollicutes*-specific probe MCP-52. The image is reconstructed by overlapping 12 different focal planes.

Epithelial cell nuclei stained with propidium iodide are marked by red spots. *Mollicutes* cells (green), presumably of '*Ca.* Phytoplasma solani', are densely located within the salivary duct. Arrows indicate the salivary duct. C) Magnification of a section of the lobe in B) showing a single focal plane with a dense colonization by *Mollicutes* cells that are confined within the salivary duct (arrows). D,E) CLSM image of FISH of salivary gland lobes with the eubacterial probe Eub338 (D), and with the Bois Noir-specific probe ph1107 (E).

Fig. 5. Localization of symbionts the gut of *H. obsoletus*. (A-D) FISH of the insect gut after hybridization with the Cy3-labeled *Sulcia*-specific probe S1150 (blue spots indicated by arrows) and the FITC-labeled CFB319 probe specific for Bacteroidetes (green) (A-B) allows to analyse the differential distribution of bacteria in the gut. The images A and B reconstruct the entire insect gut, shown in the interferential contrast micrograph in C). Epithelial cell nuclei are stained with propidium iodide (red). In D), the magnification of a portion of the gut (indicated by the white rectangle in A) shows the presence of several clusters of distinct *Sulcia* cells (indicated by asterisks). (E-H) FISH of the midgut of *H. obsoletus* with the probes specific for *Cardinium* E), for the HO1-V symbiont (F), and for *Purcelliella* (G). In H) the intestine pictured by interferential contrast is shown.

Fig. 6. Symbiont localization in the gonads of *H. obsoletus*. A-D) Images of an insect ovary pictured by interferential contrast microscopy (A) and CLSM, after staining with propidium iodide (B) and FISH with the FITC-labeled Eub338 probe specific for Bacteria (C) and the Cy3-labeled probe S1150 specific for the *Sulcia* (D). Arrows in (C) and (D) indicate the ovary duct densely colonized by bacteria other than *Sulcia*. E-F) Magnification of an oocyte (labeled with plus) and a nurse cell (asterisk) present in panels A-D. Superposition of the interferential contrast microscopy images and the FISH images are reported. Arrows indicate zones,

corresponding to the follicular cells, with hybridization signals of the Eub338 probe but not of the S1150 probe. G-H) Interferential contrast micrograph of a ovary portion (G) and CLSM image of FISH with the *Wolbachia*-specific probes W1 e W2 (H). A specific localization of these bacteria in the follicles is shown. I) Transmission electron microscopy image of a follicle, showing the interface between the oocyte (oo) and the follicular cell (fc). Different symbiont cell morphotypes are present in the oocyte and the follicular cell. Those in the follicular epithelium are probably *Wolbachia*, while bacterial cells in the oocyte showed the strap-like cell shape typical of *Sulcia*. J) Detail of the follicular cell cytoplasm showing the typical brush-like structure (arrow) of '*Ca.* Cardinium hertigii' K-M) Image of an ovaric egg showed as interferential contrast picture (K), and CLSM image of the hybridization with the *Sulcia*-specific probe S1150 (L) and the HO1-V-specific probe V370 (M). N) Superposition of the FISH images over the interferential contrast microscopy image of a male reproductive system, hybridized with the FITC-labeled Eub338 probe specific for Bacteria (green) and the Cy3-labeled probe HOS1150 specific for the HO1-V symbiont (blue). O-R) Interferential contrast (O) and CLSM images of a male reproductive system hybridized with the *Sulcia*-specific probe S1150 (P), with the *Purcelliella*-specific probe P820 (Q), or the HO1-V-specific probe V370 (R). The different organs of the male reproductive system are indicated by arrows. In N) testes (white arrows) show the signal of the S1150 probe specific for *Sulcia*, while accessory glands (black arrows), hybridized with the bacterial probe Eub338, indicate the presence of bacteria other than the HO1-V symbiont. In P-R), while the testes (white arrows) hybridized with all of the probes, accessory glands show a very weak signal after hybridization with both the *Sulcia*- and *Purcelliella*-specific probes (P, Q). On the other hand, FISH with the HO1-V probe show a strong signal (R).

Fig. 7. Visualization with CSLM of the gut of nymphs and female gonads of *H. obsoletus*. A) Interferential contrast microscopy image of a female gonad. B and C) DAPI staining and FISH with the Cy5-labeled W1 probe specific for *Wolbachia* (yellow). Insect cell nuclei stained with DAPI are coloured in blue. Magnifications of an immature ovariole (asterisk) (B) and of a mature egg (plus) (C) are shown. D) Interferential contrast microscopy image of a nymphal gut overlapped with a FITC-labeled W2 probe specific for *Wolbachia* (green). E) The same image after propidium iodide staining and FISH using the FITC-labeled probe W2 specific for *Wolbachia* (green).

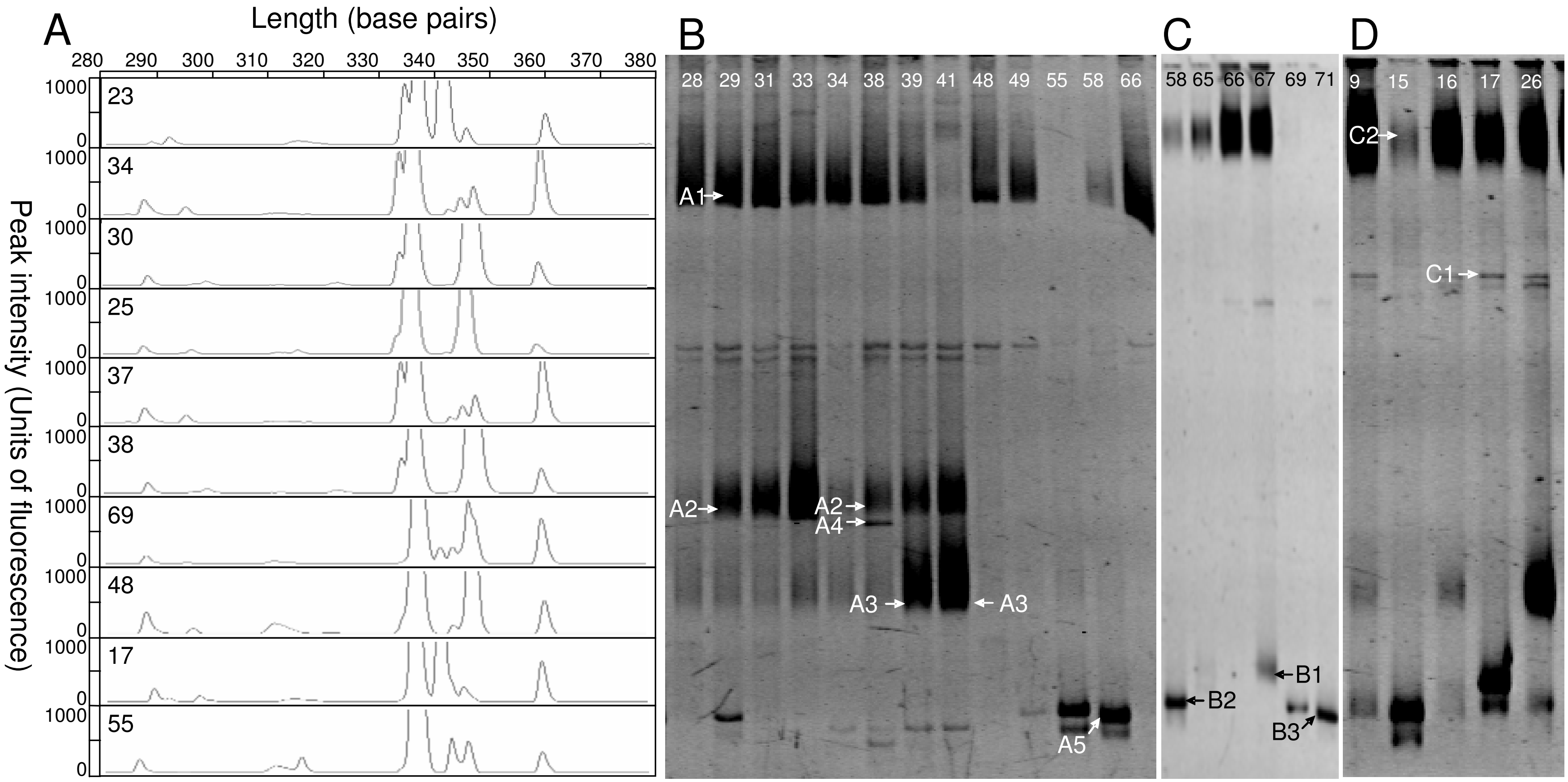


Fig. 1. - Gonella et al. 2010 - Ms.: "Bacterial endosymbiont localization......."

Fig. 2. - Gonella et al. 2010 - Ms.: “Bacterial endosymbiont localization....”

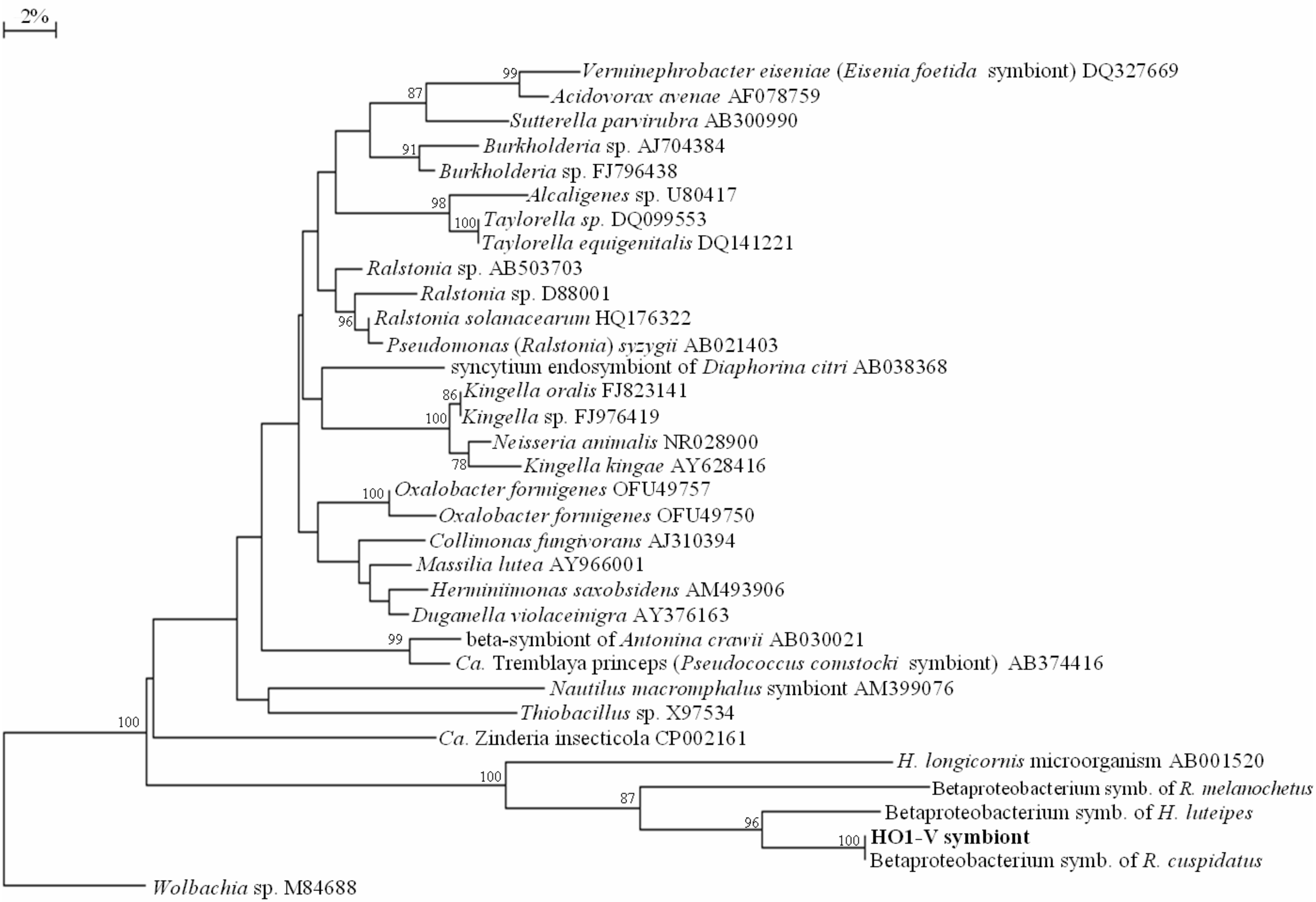


Fig. 3. - Gonella et al. 2010 - Ms.: “Bacterial endosymbiont localization.......”

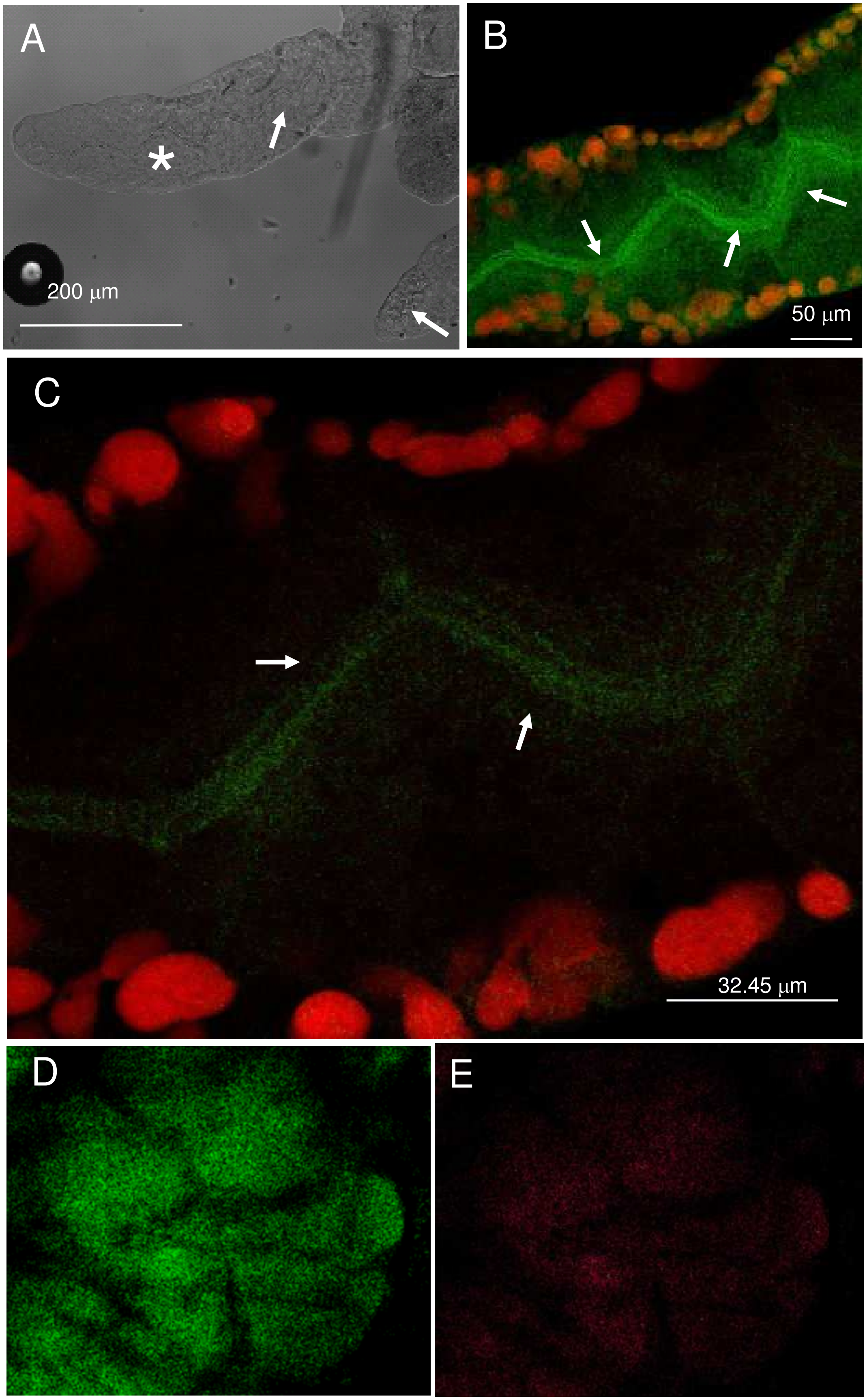


Fig. 4. - Gonella et al. 2010 - Ms.: “Bacterial endosymbiont localization.......”

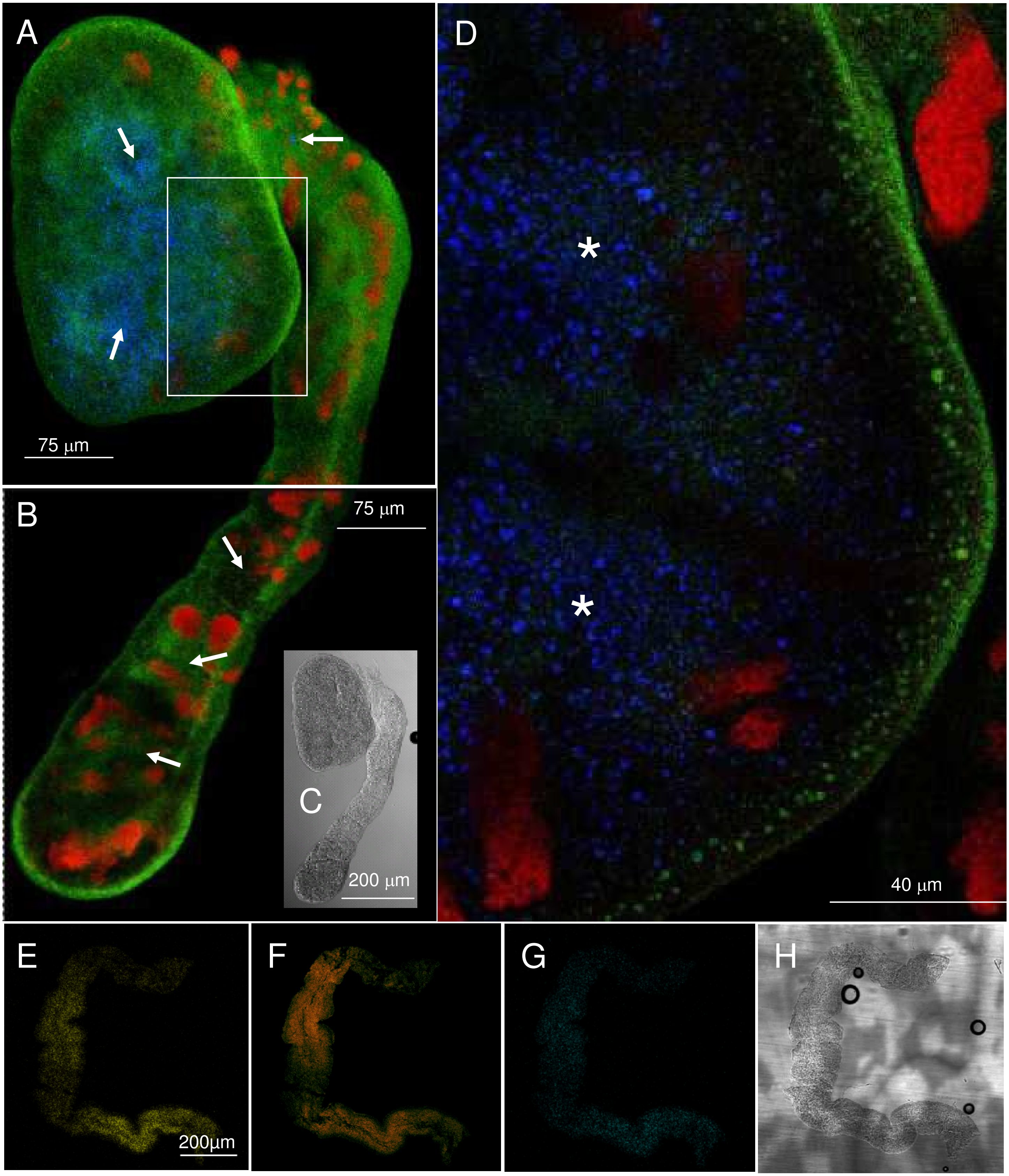


Fig. 5. - Gonella et al. 2010 - Ms.: “Bacterial endosymbiont localization.......”

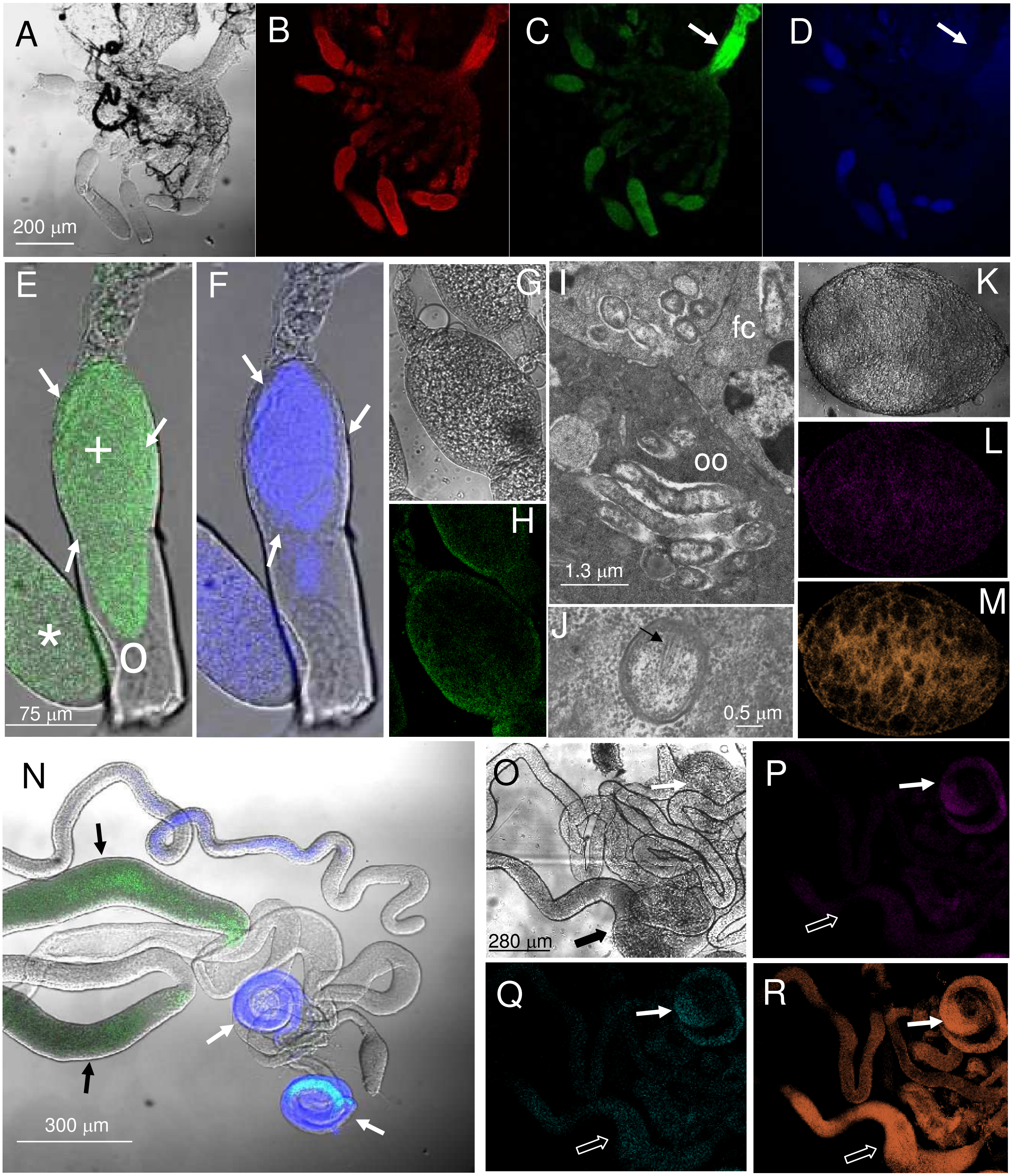


Fig. 6. - Gonella et al. 2010 - Ms.: "Bacterial endosymbiont localization......."

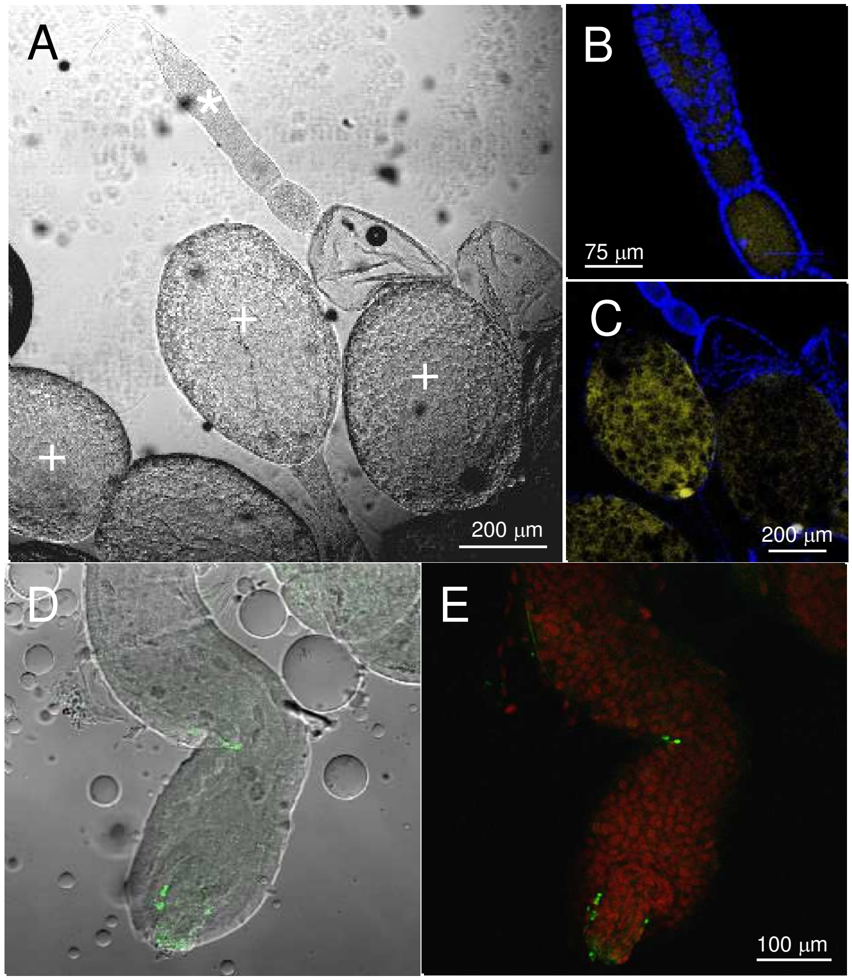


Fig. 7. - Gonella et al. 2010 - Ms.: “Bacterial endosymbiont localization.......”

Responses to the Editor’s comments:

(1) The redundancies between the Results and Discussion section must be eliminated. Thus, these sections need to be condensed. I am recommending that the combined length of these two sections should not exceed 10 pages (currently 12.5 pages). Per comments from the reviewers, combining these two sections might be advantageous.

To eliminate the redundancies between the Results and Discussion sections they have been condensed in a single part, which is included in less than 10 pages as required.

(2) The use of color in Figures 1-3 is not justified. Please convert to black and white. (3) The text quality in Figures 2 and 3 is quite low. The zigzag underlining of names hinders legibility and should be deleted. Likewise, the text is very ragged and difficult to read. Please improve legibility.

Figures 1-3 have been improved in quality, by converting them from color to black and white, and by reducing the total number of branches of the phylogenetic trees, in order to enhance the legibility without loosing significance.

Responses to the Reviewers' comments:

REVIEWER 1:

General comments: The authors have satisfactorily addressed the comments raised in the previous version of the manuscript and have done a lot of additional work in order to unravel and characterize the symbiotic diversity associated with *Hyalesthes obsoletus*; however the presentation could be further improved. Methods are long with some details, which could be removed providing reference, or put in the supplementary materials. Results are very long (particularly the first two sections), with many details which could be omitted since the figures and tables provided are very comprehensive. In addition, Results and Discussion look alike in some parts. I suggest authors to combine them.

Methods have been shortened by substituting details with references when possible. Results and Discussion have been condensed, reduced and combined in a single section (see also the answer to Editor’s comment 1).

Minor comments:

(a) Page 2, line 13: Change “which” to “with”

The word was changed in the text (page 2, line 13 of the new manuscript version)

(b) Page 2, line 17 (and throughout the manuscript): change “Candidatus Cardinium” to “Candidatus Cardinium hertigii”

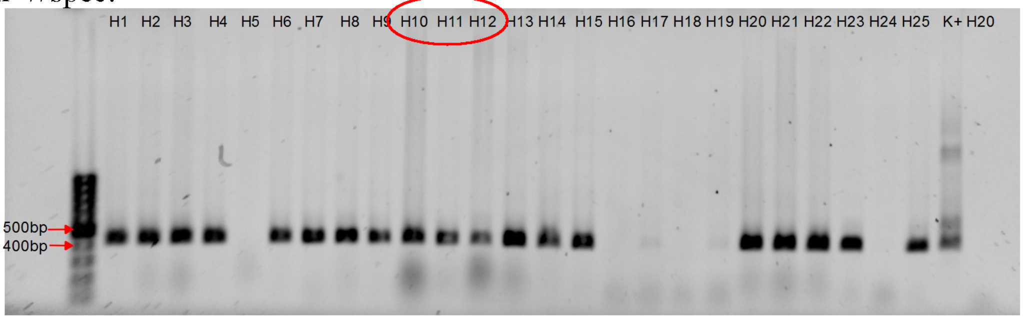
The expression has been changed in the text.

(c) Page 7, lines 4-5: wsp gene PCR primers often provide false positive results. I suggest authors to use recently designed universal 16S rRNA gene primers, WspecF and WspecR.

We thank the reviewer for the suggestion; we tried the primers WspecF/R in order to assess the viability of our data. We carried out two concurrent PCRs with the new primer pair and the wsp ones respectively, on 25 of the 90 specimens employed in this study, in order to better compare the results. Positive samples were the same in both cases, with the exception of three samples that were positive with the Wspec primers and negative with the wsp pair. For this reason we decided to maintain our data in the text and in Table 3.

Primer pair wsp:

Primer pair Wspec:



(d) Page 10,lines 17-19: The 16S rRNA gene PCR primers reported in Heddi et al. (1999) are neither newly designed primers nor are any longer universal; they may have missed Wolbachia diverse strains.

We agree with this comment; moreover we can presume that we did not miss positive signals in FISH experiments. Indeed, the *Wolbachia* strain of *H. obsoletus* was actually hybridized with the reported probes; moreover we did not detect more than one strain nor in DGGE profiles or in sequencing amplicons obtained with the specific primers.

REVIEWER 2:

Comments:

This paper describes a complex set of symbiont associations in individuals of a planthopper species that vectors a phytoplasma disease in Italian vineyards.

Authors use a variety of 16S rRNA assays, plus FISH based on these retrieved sequences, to determine the presence and distribution in the body of these symbionts. Most of the reported associations were already known: Sulcia and Purcelliella were described from cixiids including this species by Bressan et al. 2009. Wolbachia and Cardinium were reported in another study and are widespread in insects. The phytoplasma itself is already known in this vector.

The major new results are the report of the presence of the Sulcia symbiont in the gut rather than a bacteriome, the absence of bacteriomes, and the report of a novel symbiont lineage from the Betaproteobacteria.

A significant contribution of this paper would be the description of the location of symbionts in the body of the host, with extensive FISH microscopy.

In this regard, it is odd that bacteriomes could not be found in these insects. Bacteriomes were reported by Bressan et al. for cixiids, and Sulcia and Purcelliella were reported to live in separate portions of the bacteriomes. Instead these authors report that Sulcia is in the gut, as illustrated by FISH in their Fig. 5. This is very interesting and unexpected. But when I look at Fig. 5, which is said to show an entire gut, it looks a lot like a bacteriome, leaving me somewhat uncertain. It is difficult to identify the structure out of the context of the insect, but it seems to have blind ends, for example.

Actually the structure in figure 5 does not have blind ends, but it is an artefact due to tissue winding on the slide after hybridization. Focussing at microscope, the upper end is actually cut due to dissection, whereas the bottom end is below the gut itself showing a false blind end.

Fig. 5 is a nice picture, and a key part of this paper, but it is not well explained. The legend states that one probe is for Sulcia and another for Bacteroidetes which includes Sulcia and Cardinium. Does this mean the blue indicates Sulcia and the green indicates Cardinium? This is not explained in the text or the legend. Where is Purcelliella? This is not shown in the micrographs presented or discussed in the text.

We are grateful for these suggestions about fig. 5; nevertheless we did not state that the green signal is *Cardinium* because we did not use a *Cardinium*-specific probe in that hybridization. Since we had a high number of probes for each bacterium and for controls (Eubacterial, Mollicutes, Bacteroidetes), we were not able to combine the hybridizations of all of the symbionts in the same tissues. We performed other hybridizations with the *Cardinium*-specific probes, as well as the *Purcelliella*-specific probe (that is not shown in the same organ as *Sulcia* for the same reason) in other midgut sections, and in both cases we obtained a positive signal. To improve the manuscript according with the Reviewer's suggestion, we added the images showing these hybridizations in Fig. 5. Moreover, a speculation on the possible co-localization within the same midgut between *Sulcia* and *Cardinium* has been added in the text, to clarify the picture's explanation (page 14, lines 3-6 in the new manuscript version).

An implication would be that this particular host species lacks bacteriomes whereas other species of Cixiidae possess them. Is this what the authors are implying? This needs more direct examination, as it is not expected.

We agree this is an important issue to be pointed out. We did not write in the text that *H. obsoletus* lacks the bacteriome because we can not demonstrate this absence, and we agree with the reviewer that it is not expected. We just did not find any organs recognizable as bacteriomes in the individuals we dissected and employed for tissue analysis.

Another important potential contribution of the paper is the report of a new Betaproteobacterium that appears to be abundant in individual hosts and high in prevalence in this host species. This organisms has not been surveyed in other hosts and there is a lack of detailed microscopy, so it is a rather minimal report for a Candidatus name.

We appreciated the reviewer's suggestions on this point. To support the proposal for the Candidatus name we enlarged the screening with HO1-V specific primers to other cixiid species, as proposed by the reviewer. A positive signal by specific PCR was found in three species, *Hyalesthes luteipes*, *Reptalus cuspidatus* and *Reptalus melanochetus*. Moreover the 16SrRNA gene sequences of the HO1-V-like symbionts of these insects clustered together with the *H. obsoletus* HO1-V sequence in a separated branch from any other symbiotic or free-living relatives. We believe that these evidences strengthen our proposal, and we described these additional data in the text (page 7, lines 13-15; page 18, lines 1-4 in the new manuscript version; Fig. 3). Concerning the lack in microscopy, to support our proposal for the Candidatus name we added further FISH images showing the localization of the HO1-V symbiont as suggested (Fig. 5 E-H)

The paper could be shortened as it is includes some repetition. For example in the introduction, a very brief mention of the vector status of the insect would be enough.

The Introduction section has been consistently reduced, by removing redundant information.

In Results, the exact numbers of individuals do now have to be given for every PCR assay, as these numbers are in Table 3 and are more readily seen there.

The numbers of individuals have been removed from the text, maintaining only the infection percentages that are not included in Table 3.

Sometimes the Results repeats the Methods, for example, on p 12 the following could be deleted: "The prevalence of 'Ca. Phytoplasma solani', Wolbachia, Cardinium, 'Ca. Sulcia muelleri', 'Ca. Purcelliella pentastiridorum', and HO1-V symbiont in H. obsoletus was studied on the DNA extracted from a total of 80 individuals by using PCR assays targeting the 16S rRNA gene with symbiont-specific primers (Table 3). The identity of the amplified DNA was confirmed by sequencing some of the amplified fragments." This information was given earlier in the Methods. This is just an example; similar repetition is found in other parts.

The redundant parts in the Results and Discussion sections have been eliminated during the text reorganization; in addition the following paragraphs have been changed: "The bacterial community associated to *H. obsoletus* from BN-contaminated areas was studied by means of LH-PCR, targeting a portion of the 16S rRNA gene. LH-PCR discriminates bacterial species according to length polymorphisms in the 16S rRNA genes." was changed to "The bacterial community associated to *H. obsoletus* from BN-contaminated areas was studied by means of LH-PCR." (page 8, lines 23-25; page 9, line 1 in the new manuscript version). "The prevalence of 'Ca. Phytoplasma solani', Wolbachia, Cardinium, 'Ca. Sulcia muelleri', 'Ca. Purcelliella pentastiridorum', and HO1-V symbiont in H. obsoletus was studied on the DNA extracted from a total of 80 individuals by using PCR assays targeting the 16S rRNA gene with symbiont-specific primers (Table 3). The identity of the amplified DNA was confirmed by sequencing some of the amplified fragments." Was changed to "The prevalence of '*Ca.* Phytoplasma solani', *Wolbachia*, *Cardinium*, *'Ca.* Sulcia muelleri', '*Ca.* Purcelliella pentastiridorum', and HO1-V symbiont in *H. obsoletus* was studied by PCR assays targeting the 16S rRNA gene with symbiont-specific primers (Table 3)." (page 12, lines 6-9 in the new manuscript version).

Minor comments:
Page 6
Diagnostic PCR is described and the paper reports negative PCR reactions as results. But no positive control reactions are described, or controls for the DNA template quality. Are negatives meaningful? It is surprising to find Sulcia and Purcelliella absent from some individuals, but a failed PCR does not really indicate absence. If such controls were not performed it should be pointed out that he negatives may not indicate absence. Perhaps this is

implied by the wording which gives infection rates as "miminum" rates, but it could be stated more directly.

The total DNA amount and quality has been checked by spectrophotometer quantification, and a positive control was included in each PCR to exclude unsuccessful reactions. This has been specified in the text by including the following sentence in the Methods section: "Each PCR assay included a cloned amplicon sample specific for each microorganism as positive control and a water sample as negative control." (<u>page 7, lines 6-8 in the new manuscript version</u>). The "minimum" expression about the infection rates rule out possible single-sample failed reactions.

Page 11
This sentence needs rewording:
"The almost entire 16S rRNA sequence of this bacterium, obtained by combining specific and universal primers, was incorporated in the branch of the Gammaproteobacteria phylogenetic tree including Purcelliella symbionts of the genus Hyalesthes and related (Fig. 2B)."

The sentence has been changed to "The almost entire 16S rRNA sequence of this bacterium, obtained by combining specific and universal primers, was incorporated in the branch of the Gammaproteobacteria phylogenetic tree that includes *Purcelliella* symbionts of the genus *Hyalesthes* and other cixiids (Fig. 2B)" (<u>page 11, lines 3-7 in the new manuscript version</u>).

P 12
Acyrtosiphon misspelled.

"*Acyrtosiphon*" has been changed to "*Acyrthosiphon*"

P 18
There could be dependence of two coevolving symbionts even if they are in different parts of the insect host. Metabolites can be exchanged through the insect hemolymph. Therefore the last part of the following passage is questionable: "The recruitment of symbionts successively to the establishment of Sulcia has been proposed for other Auchenorrhyncha (42), and the possible co-diversification of different bacteria, with the development of a complementarity, can be assumed. Such a process could have taken place between Sulcia and Purcelliella, however in other cixiid models these two symbionts were reported to reside in different specialized organs, with a possible exclusion of the codependence between the bacteria (12)."

This part has been consistently changed in the text reorganization, and the last sentence has been eliminated (<u>page 15 in the new manuscript version</u>).

Table 2
Use "H01-V" in table for the Betaproteobacterium as this is how it is referenced through the text.

The organism name in that table is referred to the closest relative whose Accession number is indicated in the following column, and the correct name of this organism is the one indicated in the table. Moreover, in the Result section, the name of the most relative sequence is indicated, just before designating our symbiont as HO1-V (<u>page 10, lines 22-25; page 11, lines 1-2 in the new manuscript version</u>).

Fig. 2. This tree for Gammaproteobacterial symbionts only includes symbiotic lineages except for a couple of species. It is a little misleading because it appears that a large clade is

symbiotic, when there would be non symbionts mixed into the clade if they had been included in the analysis. For example, free-living Serratia or Yersinia species could be included to give a more balanced picture, showing that these symbionts reflect multiple origins of symbiosis.

Fig. 2 has been modified by including different symbiotic and free-living Gammapreotobacteria, including the genera suggested by the reviewer.

Fig. 3. It would be useful to also include other symbiotic species of Betaproteobacteria, which would presumably occupy different positions in this tree. These include Trembleya or mealybugs and the syncytium symbiont of Diaphorina citri.

Also Fig. 3 has been changed to add symbiotic Betaproteobacteria, as well as other *Vidania* sequences from other cixiid species.

REVIEWER 3:
Comments:
It appears that the authors have sufficiently addressed the concerns and criticisms raised by the previous 4 reviewers. The authors have added new data, revised and condensed the manuscript, and improved the clarity and writing. The data presented here are novel and a nice contribution to the field of symbiosis.

We appreciate the positive feedback and we wish that the new manuscript version represent a further improvement.